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Control of Anticoagulant Therapy with a Chromogenic Substrate

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Abstract. Prothrombin is determined with the aid of a recently developed assay, based on the amidolysis of a chromogenic substrate. The assay proved to be reliable when it was compared with more conventional coagulation assays in the control of oral anticoagulant therapy, both in the therapeutic range and in a case of overdosage. As is the case in coagulation tests, heparin therapy remains a disturbing circumstance. The prothrombin concentration was measured (a) in the plasma of 50 long-term anticoagulated patients, and the results were compared with those obtained with a one-stage coagulation assay and with those obtained with Thrombotest® determinations, and (b) during vitamin K administration in the plasma of a patient with a severe intoxication of a vitamin K antagonist.

Introduction

Recently, we developed a method for the determination of prothrombin with the use of a chromogenic substrate (5). In this method, prothrombin is first activated with human factor X_a and, in a second stage, the thrombin concentration is determined by its amidolytic activity towards a synthetic substrate (Chromozym® TH). From this substrate, thrombin liberates the yellow-coloured *p*-nitroanilide. The thrombin concentration is proportional to the generation rate of *p*-nitroanilide which can be recorded spectrophotometrically and is expressed as absorbancy change per unit of time. Here we present results concerning the practical value of this assay for routine prothrombin determination.

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Materials and Methods

Buffers. Buffer A: 0.075 *M* NaCl, 0.075 *M* imidazol, 0.075 *M* tris-HCl, pH 8.4. Buffer B: 0.087 *M* NaCl, 0.0029 *M* sodium acetate, 0.0029 *M* sodium barbital, pH 7.4.

Reagents. Thrombotest® was obtained from Nyegaard (Norway). Heparin, 5,000 U/ml, from Organon (The Netherlands), Chromozym TH from Boehringer, Mannheim (FRG) and Taipan snake venom from Sigma (USA).

Plasma and Coagulation Factors. Plasma was prepared by collecting blood in 0.13 *M* trisodium citrate (10% v/v) and separated from the cells by centrifuging for 10 min at 2,000 *g*. Factor X_a was prepared as described before (5), and bovine thrombin was obtained as Topostasine® from Roche (Switzerland) dissolved in buffer B to a concentration of 50 NIH U/ml and stored at −20 °C. Antiprothrombin was prepared as described by *Bas et al.* (2).

Prothrombin Determination. (1) One-stage coagulation assay as described by *Koller et al.* (6), modified in such a way that only human material was used. (2) Two-stage method with the aid of Chromozym TH as described earlier (5). (3) One-dimensional electrophoresis according to *Laurell* (8).

Results

Determination of Prothrombin in Anticoagulated Patients

Plasma was obtained from 50 patients randomly chosen from the local thrombosis service, and the prothrombin concentration was measured with the one-stage coagulation assay and the chromogenic substrate assay. The relation between the values obtained in these tests is shown in figure 1. The level of significance (p) of the correlation between these methods was 0.05 (t test). With the routinely used Thrombotest, a positive correlation of 0.69 was found in the range 5–15 of the Spearman rank correlation coefficient. The determination of prothrombin in a case of vitamin K antagonist intoxication presents a test case to exclude the possibility that decarboxyprothrombin (Pivka-II) is coestimated in our tests.

In the case at hand, the Thrombotest and the concentration of prothrombin, factor VII and factor X (all measured with the one-stage coagulation assay) were below 5% of the normal values. The increase of the prothrombin level after a single oral dose of 30 mg vitamin K was assayed with the method using chromogenic substrates. The results are shown in figure 2. The results were similar to those obtained with the one-stage coagulation assay. Immediately after administration of vitamin K, a sharp increase of the prothrombin concentration was observed, which leveled off after 2 more days.

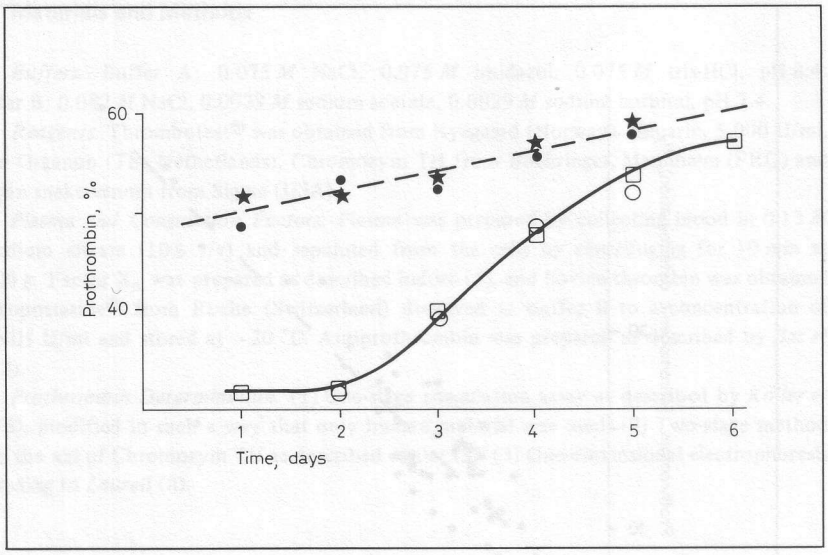

Fig. 2. Time course of the prothrombin generation in plasma after vitamin K antagonist intoxication. Blood was collected at the first 6 days of hospitalization. The 2nd day, 30 mg of vitamin K were given orally, immediately after collecting the blood. Prothrombin was assayed either with a one-stage method (○) or with the aid of a chromogenic substrate. In the latter case, prothrombin was activated either with factor X_a (□) or with Taipan snake venom (●) or with staphylocoagulase (★).

antigenic determinants. No significant differences were observed between these results and those shown in figure 2.

In current practice, heparin therapy interferes with the results of coagulation factor determination. This is also the case with this method using chromogenic substrates. Nevertheless, in the presence of small therapeutic amounts of heparin in the plasma, small amounts of prothrombin will be determined correctly (fig. 3).

Discussion

A recently developed method for the determination of prothrombin was tested in 50 patients, who were on oral anticoagulation therapy. The correlation between the new assay and the one-stage prothrombin assay was good. The

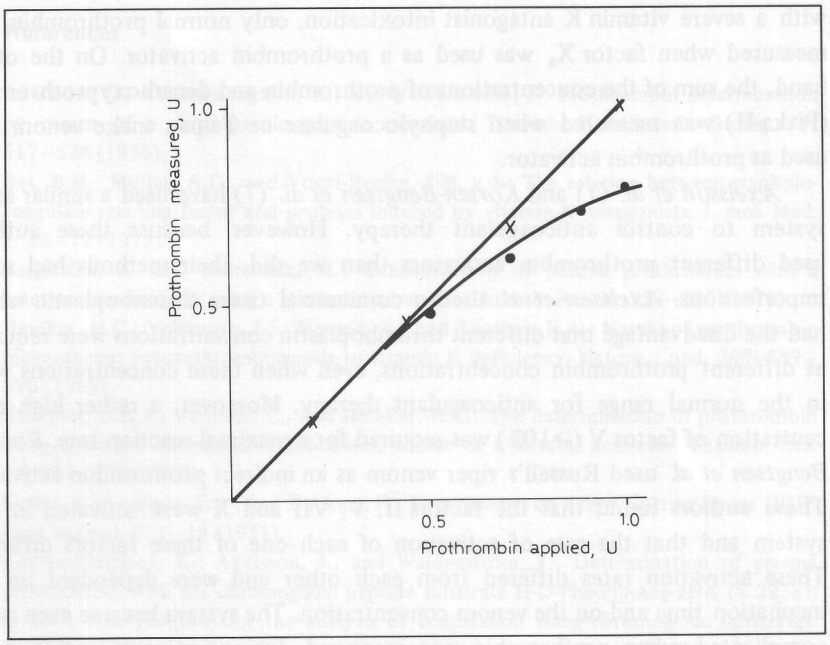

Fig. 3. Increase of the prothrombin concentration in normal plasma as a function of the plasma dilutions in the presence (•) or absence (x) of 0.25 U heparin/ml plasma. For further details, see Materials and Methods.

correlation between the data obtained with the routinely used Thrombotest assay and those obtained with specific prothrombin assays is moderate due to the fact that the Thrombotest is a non-specific assay in which the clotting time is dependent on the concentration of prothrombin as well as that of several other coagulation factors.

Assays in which prothrombin is detected with the aid of a chromogenic substrate have three advantages over the conventional coagulation assays: (a) the assay can be easily adapted to automatic measurements; (b) the conformation of fibrin as the end point of the reaction is avoided and hence the conditions interfering with fibrin formation will not disturb the test (3), and (c) the conditions of the test can be more strictly standardized than those of a coagulation test.

As can be seen from the experiments in which the new prothrombin assay was used to register the prothrombin concentration in the plasma of a patient

with a severe vitamin K antagonist intoxication, only normal prothrombin was measured when factor X_a was used as a prothrombin activator. On the other hand, the sum of the concentrations of prothrombin and decarboxyprothrombin (Pivka-II) was measured when staphylocoagulase or Taipan snake venom was used as prothrombin activator.

Axelsson et al. (1) and *Korsan-Bengtsen et al.* (7) have used a similar assay system to control anticoagulant therapy. However, because these authors used different prothrombin activators than we did, their methods had some imperfections. *Axelsson et al.* used a commercial tissue thromboplastin which had the disadvantage that different thromboplastin concentrations were required at different prothrombin concentrations, even when these concentrations were in the normal range for anticoagulant therapy. Moreover, a rather high concentration of factor V ($\geqslant$10%) was required for a maximal reaction rate. *Korsan-Bengtsen et al.* used Russell's viper venom as an indirect prothrombin activator. These authors found that the factors II, V, VII and X were activated in this system and that the rate of activation of each one of these factors differed. These activation rates differed from each other and were dependent on the incubation time and on the venom concentration. The system became even more complicated when prothrombin was measured during anticoagulant therapy, because also the decarboxyfactors II, VII and X are slowly activated and hence decarboxyfactor II is partially coestimated.

With the modified method which we used in our investigations, these types of shortcomings could be avoided because (a) decarboxyfactor II is not coestimated, (b) the reaction occurs in the absence of thromboplastin, (c) only 1% of factor V is required and (d) the only prothrombin activator is factor X_a which is added to the samples in excess.

As the occurrence of decarboxyprothrombin is pathogenic for vitamin K deficiency (4), this test allows to diagnose a vitamin K deficiency instantaneously. This may be of importance, not only to spot an intoxication quickly, but also to recognise the contribution of vitamin K deficiency due to resorption defects in the low coagulation factor level found in liver disease.

From the experiments presented in this paper, we conclude that the new prothrombin assay correlates very well with the specific coagulation assays in varying types of experiments. As it is a quick and easy assay, it is fit for the routine determination of prothrombin in the plasma of anticoagulated patients and may be adapted to automatic measurements in the laboratories of clinical chemistry. It can be easily modified to a test that immediately indicates whether or not a patient has a vitamin K deficiency.

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